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Original scientific article

GENETIC DIVERSITY OF *Lonicera* L. (Caprifoliaceae) ESTIMATED BY MOLECULAR MARKERS AND MORPHOLOGICAL CHARACTERS

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Members of *Lonicera* are characterized by opposite, narrowly elliptic to obovate leaves, white, yellow, reddish, or purple-red corolla with capitate stigma and undulate calyx margin. In Flora Iranica, Wendelbo (1965) classified 19 species of the *Lonicera* into two subgenera (*Chamaecerasus* and *Lonicera*) and three sections, namely *Isoxylosteum*, *Isika* and *Coeloxylosteum.* The four studied species belong to subgenus *Chamaecerasus* and sections *Isika* and *Coeloxylosteum*. No detailed Random Amplified Polymorphic DNA (RAPD) studies were conducted to study *Lonicera* genetic diversity. Therefore, we collected and analyzed three species from 2 provinces regions. Overall, 45 plant specimens were collected. Our aims were 1) to assess genetic diversity among *Lonicera* species 2) is there a correlation between species genetic and geographical distance? 3) Genetic structure of populations and taxa. We showed significant differences in quantitative morphological characters in plant species. The Mantel test showed correlation (r=0.66, p=0.0001) between genetic and geographical distances. We reported high genetic diversity, which clearly shows the *Lonicera* species can adapt to changing environments since high genetic diversity is linked to species adaptability. Present results highlighted the utility of RAPD markers and morphometry methods to investigate genetic diversity in *Lonicera* species.

Keyword: Gene flow, Random Amplified Polymorphic DNA (RAPD), *Lonicera* species, isolation, morphometry

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INTRODUCTION

Genetic diversity is a vital feature that helps plant species survive in an ever-changing environment, and it sheds light on understanding the phylogenetic affinity among the species (ERBANO *et al*., 2015). Quite a significant number of genetic resources and materials programs of plant species have been carried out to preserve the plant species worldwide. Scientific data indicate that genetic diversity plays a pivotal role in conservation programs (GOMEZ *et al*., 2005).

To better understand genetic diversity, the biologist study population size. Population size is considered one of the central factors to understand the variability in a gene (ELLEGREN and GALTIER, 2016; TURCHETTO *et al.*, 2016). Genetic variation and diversity are essential for species to survive because individuals are separated due to genetic or geographical barriers, often resulting in scattered populations. Since these individuals have limited gene flow, there is a greater chance of a decline in population size (FRANKHAM, 2005). Given the significance of genetic diversity in conservation strategies, it is of utmost importance to disentangle genetic diversity in plant species, particularly threatened and rare species (CIRES *et al*., 2013, ESFANDANI-BOZCHALOYI *et al*., 2018a, 2018b, 2018c, 2018d).

Lonicera L. (Caprifoliaceae) includes more than 200 species (MABBERLEY, 2008) worldwide, with 19 species in the region of Flora Iranica (WENDELBO 1965). The genus is mainly distributed in temperate to subtropical regions of the northern hemisphere: Europe, Russia, East Asia, and North America (HSU and WANG, 1988; MABBERLEY, 2008). In the flora of Iran, the genus *Lonicera* is represented by nine species (KHATAMSAZ, 1995; GHAHREMANINEJAD and EZAZI, 2009) across the north, northwest and northeast of the country. Some species are medicinal plants (ZENG *et al*., 2017). Dried *Lonicera* flowers and buds are known as Flos Lonicera and have been a recognized herb in the traditional Chinese medicine for more than 1500 years (LI *et al*., 2015). It has been applied for treatment of arthritis, diabetes mellitus, fever, and viral infections (SHANG *et al*., 2011; LI *et al*., 2015). The plants are erect shrubs, occasionally small trees. Members of *Lonicera* are characterized by opposite, narrowly elliptic to obovate leaves, white, yellow, reddish, or purple-red corolla with capitate stigma (JUDD *et al*., 2007), and undulate calyx margin. In Flora Iranica, WENDELBO (1965) classified 19 species of the *Lonicera* into two subgenera (*Chamaecerasus* and *Lonicera*) and three sections, namely *Isoxylosteum*, Isika and *Coeloxylosteum.* The four studied species belong to subgenus *Chamaecerasus* and sections *Isika* and *Coeloxylosteum*.

Molecular data have been obtained in phylogenetic studies and species divergence researches (KAZEMPOUR OSALOO *et al*., 2003; 2005). These data can also provide supportive and extra criteria for systematic classification of the studied species that have been based only on the morphological characters (CHASE *et al*., 1993). The internal transcribed spacer (ITS) is the region of the 18S-5.8 S-26S nuclear ribosomal cistron (BALDWIN *et al.*, 1995). The spacers contain the signals needed to process the rRNA transcript (BALDWIN, 1992; BALDWIN *et al*., 1995) and have often been used for inferring phylogeny at the generic and infrageneric levels in plants (e.g. BALDWIN, 1992; BALDWIN *et al*., 1995; KAZEMPOUR OSALOO *et al*., 2003; 2005). THEIS *et al*. (2008) studied phylogenetics of the *Caprifolieae* and *Lonicera* (*Dipsacales*) on the basis of nuclear and chloroplast DNA sequences. Their analysis indicates monophyly in *Lonicera* and highlights instances of homoplasy in several morphological characters. Molecular phylogenetics

of *Lonicera* in Japan has been studied by NAKAJI *et al.* (2015) on the basis of chloroplast DNA sequences. According to the results, circumscription of the higher taxonomic groups for the Japanese species of *Lonicera* proposed by Hara in 1983 is fundamentally acceptable. *Lonicera* is well known for its taxonomic complexity resulting from overlapping morphological characters. Genetic diversity studies are usually tapped due to molecular markers. Molecular markers are an excellent method to disentangle phylogenetic association between species and population. Among molecular methods or markers, RAPD (Random Amplified Polymorphic DNA) are sensitive to detect variability among individuals of species. RAPD method is cost-effective and can work with limited sample quantities. In addition to this, RAPD can amplify and target genomic regions with potential and several markers (ESFANDANI-BOZCHALOYI *et al.*, 2017a, b, c, d). Taxonomical systematics studies were conducted in the past to identify the *Lonicera* species. According to the best of our knowledge, there is no existing RAPD data on genetic diversity investigations in Iran. We studied 45 samples. Our aims were 1) to assess genetic diversity among *Lonicera* species 2) is there a correlation between species and geographical distance? 3) genetic structure of populations and taxa 4) are the *Lonicera* species able to exchange genes?

MATERIALS AND METHODS

Plant materials

Three *Lonicera* species were collected from different regions of Iran (Table 1). These species were studied via morphological and molecular methods. 45 plant samples (4-7 per plant species) were examined for morphometry purpose. The random amplified polymorphic DNA analysis method was limited to 45 samples. We focused on the following species *L. nummulariifolia* Jaub. et Spach; *L. bracteolaris* Boiss. & Buhse; *L. hypoleuca* Decne. According to previous references, all the species were identified.

Table 1. List of the investigated taxa including origin of voucher specimens

Taxa	Locality	Latitude	Longitude	Altitude(m)
L. nummulariifolia Jaub. et Spach	West Azerbaijan, Kaleybar	37° 07' 48 "	49° 54' 04"	165
L. bracteolaris Boiss. & Buhse	Hormozgan, Bandar Abbas	38 ˚ 52' 93"	47 ˚25' 92"	1133
L. hypoleuca Decne	Khuzestan, Behbahan	37° 07' 08"	49°54' 11"	159

Morphometry

In total 10 morphological (4 qualitative, 6 quantitative) characters were studied`. Five to ten plant specimens were randomly studied or morphological analyses**.** Data were transformed (Mean= 0, variance = 1) prior to ordination. Euclidean distance was implemented to cluster and ordinate plant species (PODANI, 2000).

Random Amplified Polymorphic DNA

We extracted DNA from fresh leaves. Leaves were dried. DNA extraction was carried out according to the previous protocol (ESFANDANI-BOZCHALOYI *et al.*, 2019). DNA quality was checked on an agarose gel to confirm the purity. We amplified the DNA with the aid of RAPD primers (Operon technology, Alameda, Canada). These primers belonged to OPA, OPB, OPC,

OPD sets. We selected those primers (10) which could show clear bands and polymorphism. Overall, the polymerase chain reaction contained 25µl volume. This 25 volume had ten mM Tris-HCl buffer, 500 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM of each dNTP; 0.2 µM of a single primer; 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany). We observed the following cycles and conditions for the amplification. Five minutes initial denaturation step was carried out at 94°C after this forty cycles of 1 minute at 94°C were observed. Then 1-minute cycle was at 52-57°C followed by two minutes at 72°C. In the end, the final extension step was performed for seven to ten minutes at 72°C. We confirmed the amplification steps while observing amplified products on a gel. Each band size was confirmed according to 100 base pair molecular ladder/standard (Fermentas, Germany).

Data analyses

We used an Unweighted pair group method with arithmetic mean (UPGMA) and Ward methods. Ordination methods such as multidimensional scaling and principal coordinate analysis were also performed (PODANI, 2000). The morphological difference among species and population was assessed through analysis of variance (ANOVA). PCA analysis (PODANI, 2000) was done to find the variation in plant population morphological traits. Multivariate and all the necessary calculations were done in the PAST software, 2.17 (HAMMER *et al*., 2001). To assess genetic diversity, we encoded RAPD bands as present and absent. Numbers 1 and 0 were used to show the presence and absence of bands. It is essential to know the polymorphism information content and marker index (MI) of primers because these parameters serve to observe polymorphic loci in genotypes (ISMAIL *et al*., 2019). Marker index was calculated according to the previous protocol (HEIKRUJAM *et al.*, 2015). Other parameters such as the number of polymorphic bands (NPB) and effective multiplex ratio (EMR) were assessed. Gene diversity associated characteristics of plant samples were calculated. These characteristics include Nei's gene diversity (H), Shannon information index (I), number of effective alleles (Ne), and percentage of polymorphism (P% =number of polymorphic loci/number of total loci) (SHEN *et al.*, 2017). Unbiased expected heterozygosity (UHe), and heterozygosity were assessed in GenAlEx 6.4 software (PEAKALL and SMOUSE, 2006). Neighbor-joining (NJ) and networking were studied to fathom genetic distance plant populations (HUSON and BRYANT, 2006; FREELAND *et al*., 2011). The Mantel test was carried out to find the correlation between genetic and geographical distances (PODANI, 2000). As we were interested in knowing the genetic structure and diversity, we also investigated the genetic difference between populations through AMOVA (Analysis of molecular variance) in GenAlEx 6.4 (PEAKALL and SMOUSE, 2006). Furthermore, gene flow (Nm) was estimated through Genetic statistics ($G_{ST)}$ in PopGene ver. 1.32 (YEH *et al*., 1999). We also did STRUCTURE analysis to detect an optimum number of groups. For this purpose, the Evanno test was conducted (EVANNO *et al*., 2005).

RESULTS

Morphometry

Significant ANOVA results ($P < 0.01$) showed differences in quantitative morphological characters in plant species. Principal component results explained 54% variation. First component of PCA demonstrated 33% of the total variation. Leaf morphology and traits such as

calyx length, calyx width positively correlated with corolla length, corolla color (>0.7). The second and third components explained floral characters such as corolla apex, seed length and number of segment stem leaves. Unweighted pair group method with arithmetic mean (UPGMA) and principal coordinate analysis (PCoA) plots showed symmetrical results (Figure 1).

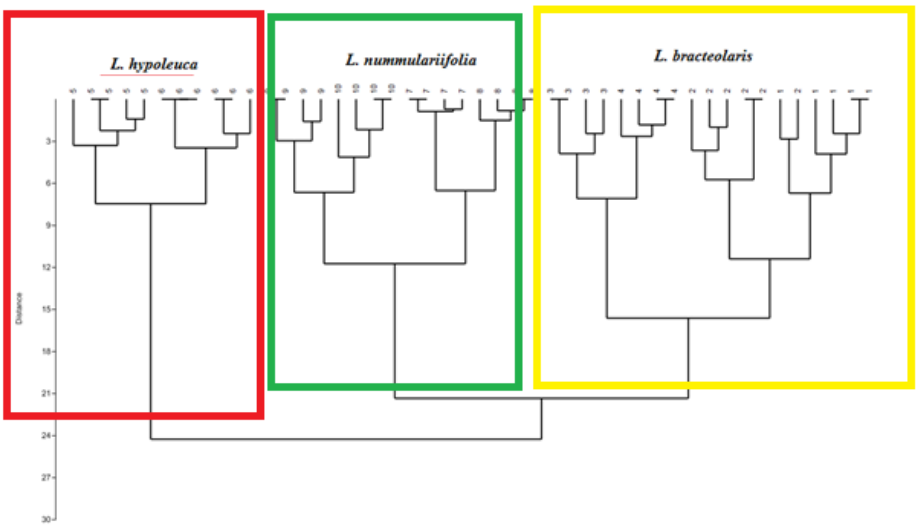

Figure 1. UPGMA clusters of morphological characters revealing species delimitation in *Lonicera* species.

Generally, plant specimens belonging to different species were separated from each other due to differences in morphology. Morphological characters divided *Lonicera* species into two groups, as evident in the UPGMA tree (Figure 1). Populations belonging to *L. hypoleuca* were in the first group. On the other hand, the second group consisted of two sub-groups. *L. nummulariifolia* formed the first sub-group. *L. bracteolaris* formed the second sub-group. These groups and sub-groups were formed due to morphological differences among the individuals of *Lonicera*. Our PCoA results also confirmed the application of morphological characters in separating and clustering the species in separate groups (Figure not included). Identical results were also reported in the UPGMA tree (Figure 1).

Species Identification and Genetic Diversity

The primers, i.e., OPC-04, OPB-01, OPA-05 and OPD-11 could amplify plant (*Lonicera* species) DNA (Figure 2). 77 polymorphic bands were generated and amplified. Amplified products ranged from 100 to 3000 bp. We recorded the highest polymorphic bands for OPA-06. OPD-03 had the lowest polymorphic bands. The average polymorphic bands ranged to

6.6 for each primer. The polymorphic information content (PIC) had values in the range of 0.44 (OPB-02) to 0.78 (OPD- 011). Primers had 0.65 average polymorphic information content values.

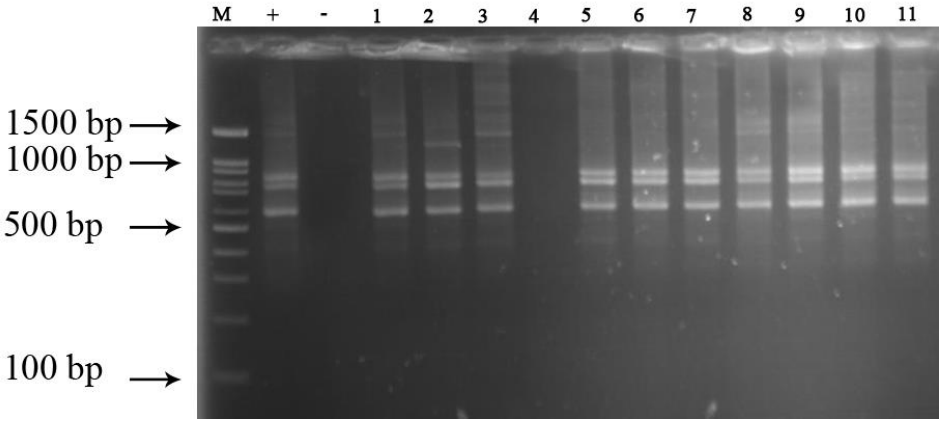

Figure 2. Gel Electrophoresis image of DNA fragments of *Lonicera* species. L = Ladder 100 bp. Arrows show polymorphic bands.

Marker index (MI) values were 4.33 (OPC-04) to 6.3 (OPB-01), with an average of 5.5 per primer. Effective multiplex ratio (EMR) values are useful to distinguish genotypes. In our study, we reported 4.3 (OPB-02) to 7.4 (OPD-08) EMR values. EMR values averaged 6.1 per primer (Table not included). All the necessary genetic features calculated of three *Lonicera* species are shown (Table 2). *L. hypoleuca* depicted unbiased expected heterozygosity (UHe) in the range of 0.047. Shannon information was high (0.38) in *L. nummulariifolia*. *L. hypoleuca* showed the lowest value, 0.055. The observed number of alleles (*N*a) ranged from 1.13 to 1.50 in *L. hypoleuca* and *L. nummulariifolia*. The effective number of alleles (*Ne*) was in the range of 1.022-1.44 for *L. hypoleuca* and *L. nummulariifolia*. Gene flow (Nm) was relatively low (0.28) in *Lonicera*.

Table 2. Genetic diversity variables of Malva (N = number of samples, Ne = number of effective alleles, I= Shannon's information index, He = gene diversity, UHe = unbiased gene diversity, P%= percentage of polymorphism in populations).

taxon	N	Na	Ne	I	He	UHe	%P
L. nummulariifolia Jaub. et Spach	12.000	1.500	1.441	0.380	0.333	0.233	50.00%
L. bracteolaris Boiss. & Buhse	13.000	1.333	1.232	0.196	0.200	0.133	33.33%
L. hypoleuca Decne	10.000	1.137	1.022	0.055	0.150	0.047	16.67%

Analysis of Molecular Variance (AMOVA) test highlighted genetic differences among *Lonicera* species (P = 0.001). AMOVA showed that 70% of genetic variation was among the species. Relative less variation (30%) was reported within the species (Table not included). Genetic similarity and dissimilarity assessed through Genetic statistics (GST) showed significant differences i.e., (0.577, P = 0.001) and D_est values (0.199, p = 0.001).

The neighbor-joining tree and PCoA plot of *Lonicera* populations based on RAPD data produced similar results therefore only PCoA plot is presented and discussed (Fig. 3). PCoA plot revealed that the three species are well differentiated on the genetic grounds. In both UPGMA and PCoA plot , samples of the *L. hypoleuca* were placed far from each other. *L. nummulariifolia* was placed close to *L. bracteolaris*, and far from *L. hypoleuca.* Genetic distance of the three species was estimated to be 3,44 by Kimura 2p distance.

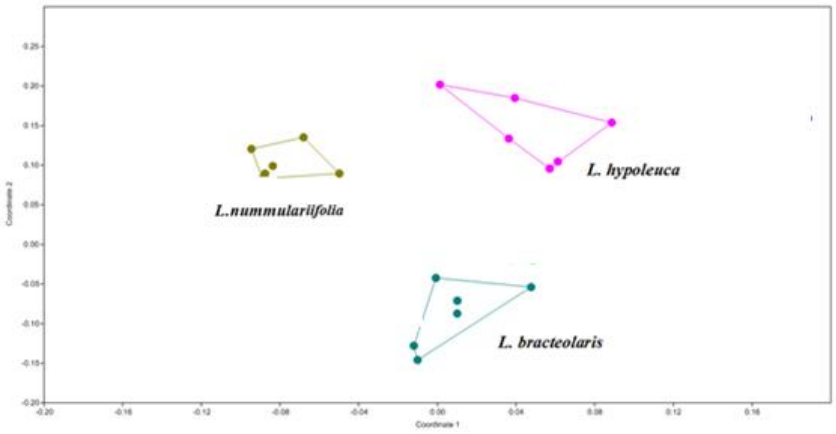

Figure 3. PCoA plot RAPD data revealing species delimitation in *Lonicera* species.

Gene flow (Nm) was relatively low (0.28) in *Lonicera* species. Genetic identity and phylogenetic distance in the *Lonicera* members are mentioned (Table 3). *L. nummulariifolia* and *L. bracteolaris* were genetically closely related (0.86) to each other. *L. bracteolaris* and *L. hypoleuca* were dissimilar due to low (0.55) genetic similarity. The mantel test showed correlation (r = 0.33, p=0.0001) between genetic and geographical distances.

Table 3. The Nei genetic similarity (Gs) estimates using RAPD markers.

pop1	pop2	pop3	
1.000			pop1
0.866	1.000		pop2
0.660	0.554	1.000	pop3

The Evanno test showed ΔK =3 (Figure not included) the genetic details of the *Lonicera* species. According to STRUCTURE analysis *L. nummulariifolia* and *L. bracteolaris* were closely related to common alleles. The rest of the *Lonicera* species are genetically differentiated due to different allelic structures. The neighbor-joining plot also showed the same result. Limited gene flow results were supported by K-Means and STRUCTURE analyses too. We could not identify substantial gene flow among the *Lonicera* species. This result is in agreement with grouping we obtained with PCOA plot (Figure 3), as these populations were placed close to each other. As evidenced by STRUCTURE plot based on admixture model, these shared alleles comprise very limited part of the genomes in these populations and all these results are in agreement in showing high degree of genetic stratification within *Lonicera* populations.

DISCUSSION

The *Lonicera* is a relatively complex taxonomic group, and several morphological characters make it difficult to identify and classify *Lonicera* species (RAY, 1995; GARCÍA *et al.*, 2009). Given the complexity, it is necessary to explore other methods that could complement the traditional taxonomical approach (ERBANO *et al*., 2015). Advent and developments in molecular techniques have enabled plant taxonomists to utilize molecular protocols to study plant groups (ERBANO *et al*., 2015). We examined genetic diversity in *Lonicera* species by morphological and molecular methods. We mainly used RAPD markers to investigate genetic diversity and genetic affinity in *Lonicera* species. Our clustering and ordination techniques showed similar patterns. Morphometry results clearly showed the utilization or significance of morphological characters in *Lonicera* species. PCoA plot results also confirmed the application of morphological characters to separate *Lonicera* species. The present study also highlighted that morphological characters such as corolla color, leaf shape, leaf length, stamens position, leaf margin and corolla lenght could delimit the *Lonicera* group. The *Lonicera* species highlighted morphological differences. We argue that such a dissimilarity was due to differences in quantitative and qualitative traits.

In our study, morphology and genetic diversity in three taxa of *Lonicera* species are given in detail for the first time. The aim of the present study was to find diagnostic features to separate species of *Lonicera* in Iran. Morphological characters are considered as an useful tool for the identification of the species, as indicated previously RAY (1995). Four species and 12 populations of the genus *Lonicera* have been studied in terms of pollen and seed micro-morphology and molecular phylogeny **(**AMINI *et al*., 2019**)**. Their results showed that micro-morphological and molecular data provide reliable evidence for differentiation of some populations from others. Since *Lonicera* systematically is a problem genus, it is necessary to use alternative methods to distinguish its taxa. Statistical evaluation of taxa can be used for taxa delimitation. The present study intends to provide further evidence for taxonomists, so as to help them in separating these three species.

PIC and MI characteristics of a primer help in determining its effectiveness in genetic diversity analysis. SIVAPRAKASH *et al.* (2004) suggested that the ability of a marker technique to resolve genetic diversity may be more directly related to the degree of polymorphism. Generally, PIC value between zero to 0.25 imply a very low genetic diversity among genotypes, between 0.25 to 0.50 shows a mid-level of genetic diversity and value ≥0.50 suggests a high level of

genetic diversity (TAMS *et al.*, 2005). In this research, the RAPD primers' PIC values ranged from 0.44 to 0.78, with a mean value of 0.65, which indicated a mid-ability of RAPD primers in determining genetic diversity among the *Lonicera* species.

In the study conducted by CHEN *et al*. (2012), 20 ISSR primers amplified 186 bands with 103 (54.63%) polymorphic bands and 58 sequence-related amplified polymorphism (SRAP) primer combinations amplified 591 bands with 347 (55.46%) polymorphic bands. Both ISSR and SRAP analyses revealed a middle level of genetic diversity in *Lonicera macranthoides* cultivars. SMOLIK *et al*. (2006) found a level of similarity for 6 populations of *Lonicera periclymenum* ranging from 82.3% to 86.6%, indicating their closely related nature. ISSR amplification was used by SMOLIK *et al*. (2010) to analyze polymorphisms of microsatellite sequences in the honeysuckle genome and to evaluate genetic diversity among 14 Polish and Russian blue honeysuckle accessions. Random amplified polymorphic DNA (RAPD) analysis was used by NAUGŽEMYS *et al*. (2011) to assess the genetic relationships among 51 accessions of blue honeysuckle. The pairwise genetic distance (GDxy) values among studied accessions ranged from 0.054 to 0.479; the mean GDxy was 0.283. Knowledge of the content of secondary metabolites in individual genotypes allows us to choose the best in *Lonicera* breeding programs in order to increase the nutritional value and health benefits.

Molecular markers (RAPD) and morphometry analysis were useful to study genetic diversity and population structure in *Lonicera* species identification. All the species had distinct genetic differentiation. Present results highlighted isolation and limited gene flow are the main deterministic factors that shape the *Lonicera* population. We also reported high genetic diversity, which clearly shows the *Lonicera* species can adapt to changing environments since high genetic diversity is linked to species adaptability.

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GENETIČKI DIVERZITET *Lonicera* L. (Caprifoliaceae) OCENJEN MOLEKULARNIM MARKERIMA I MORFOLOŠKIM KARAKTERISTIKAMA

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Izvod

Članove *Lonicere* karakterišu suprotni, usko eliptični do jajoliki listovi, beli, žuti, crvenkasti ili ljubičasto-crveni venci sa kapitelnom stigmom i valovitim rubom čaške. U Flora Iranica, Vendelbo (1965) klasifikovao je 19 vrsta *Lonicera* u dva podgenera (*Chamaecerasus* i *Lonicera*) i tri dela, i to *Isoxylosteum*, *Isika* i *Coeloxylosteum*. Nisu sprovedene detaljne RAPD studije za proučavanje genetičkog diverziteta *Lonicere*. Stoga smo prikupili i analizirali tri vrste iz 2 provincije regiona. Ukupno je prikupljeno 45 biljnih primeraka. Cilj nam je bio 1) da procenimo genetički diverzitet vrsta *Lonicera* 2) da li postoji korelacija između genetske i geografske udaljenosti vrsta? 3) Genetska struktura populacija i taksona. Pokazali smo značajne razlike u kvantitativnim morfološkim karakterima biljnih vrsta. Mantelov test pokazao je korelaciju (r = 0,66, p = 0,0001) između genetske i geografske udaljenosti. Izvestili smo o velikoj genetskoj raznolikosti, što jasno pokazuje da se vrste *Lonicera* mogu prilagoditi promenljivom okruženju, jer je velika genetska raznolikost povezana sa prilagodljivošću vrsta. Sadašnji rezultati ukazali su na korisnost RAPD markera i morfometrijskih metoda za istraživanje genetske raznolikosti vrsta *Lonicera*.

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